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Elevated Plasma Phenylalanine in Severe Malaria and Implications for Pathophysiology of Neurological Complications

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Cerebral malaria is associated with decreased production of nitric oxide and decreased levels of its precursor, L-arginine. Abnormal amino acid metabolism may thus be an important factor in malaria pathogenesis. We sought to determine if other amino acid abnormalities are associated with disease severity in falciparum malaria. Subjects were enrolled in Dar es Salaam, Tanzania (children) ($n = 126$), and Papua, Indonesia (adults) ($n = 156$), in two separate studies. Plasma samples were collected from subjects with WHO-defined cerebral malaria (children), all forms of severe malaria (adults), and uncomplicated malaria (children and adults). Healthy children and adults without fever or illness served as controls. Plasma amino acids were measured using reverse-phase high-performance liquid chromatography with fluorescence detection. Several plasma amino acids were significantly lower in the clinical malaria groups than in healthy controls. Despite the differences, phenylalanine was the only amino acid with mean levels outside the normal range (40 to 84 μM) and was markedly elevated in children with cerebral malaria (median [95% confidence interval], 163 [134 to 193] μM; $P < 0.0001$) and adults with all forms of severe malaria (median [95% confidence interval], 129 [111 to 155] μM; $P < 0.0001$). In adults who survived severe malaria, phenylalanine levels returned to normal, with clinical improvement ($P = 0.0002$). Maintenance of plasma phenylalanine homeostasis is disrupted in severe malaria, leading to significant hyperphenylalaninemia. This is likely a result of an acquired abnormality in the function of the liver enzyme phenylalanine hydroxylase. Determination of the mechanism of this abnormality may contribute to the understanding of neurological complications in malaria.

The coma of cerebral malaria (CM) is frequently accompanied by seizures and abnormalities of muscle tone and posture (23). The mechanisms of these neurological complications are unclear; however, cerebral ischemia is unlikely to be the sole explanation, as survivors are usually neurologically intact (17). Altered amino acid metabolism in response to malaria infection may contribute to disease severity. Circulating amino acids serve primarily as substrates for protein synthesis, metabolic energy (oxidation through the carboxylic acid cycle), or gluconeogenesis and ketogenesis. Importantly, certain amino acids are also substrates for neurochemical mediators, which can be increased by inflammatory stimuli. For example, gamma interferon increases metabolism of tryptophan through the kynurenine pathway, resulting in the production of the excitatory mediators quinolinic acid, kynurenic acid, and picolinic acid. These have been investigated as possible contributors to the neurologic dysfunction of CM (15).

We have previously shown that plasma levels of the nitric oxide (NO) precursor L-arginine were significantly reduced in African children with CM relative to levels in healthy controls (HC) and those with uncomplicated malaria (UM) (14). In addition, case fatality rates from CM were independently associated with the degree of hypoargininemia. These results paralleled previous work demonstrating similarly reduced systemic levels of NO metabolites and NO synthase expression in blood mononuclear cells from children with CM relative to those of patients with UM and controls (1). In extending our amino acid analysis from those previous studies, we discovered abnormalities in plasma phenylalanine levels in children with malaria. Additionally, we measured phenylalanine levels in plasma collected from Indonesian adults with severe malaria (SM) and UM. Here, we describe our findings and discuss how they may relate to the neurological complications of CM.

MATERIALS AND METHODS

Subjects. Plasma samples analyzed in this study were collected from a previous study of NO and malaria involving children aged between 6 months and 7 years from Dar es Salaam (1), Tanzania. Since publishing plasma arginine levels in Tanzanian children with malaria (14), we have located additional stored plasma samples (19 HC, 19 UM, and 13 CM samples) from the original cohort of children (1). These samples were included for analysis in the present study.

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TABLE 1. Characteristics of subjects in Tanzanian study groups upon enrollment[a]

Variable	HC (n = 38)	UM (n = 36)	CM (n = 51)
Age (yr)	4.6 ± 2.3	3.4 ± 2.0	3.6 ± 1.9
% Male	63	56	53
Wt (kg)	15.0 ± 4.8	12.5 ± 3.8	12.6 ± 3.5
Time since last meal (h)	12.6 ± 1.1	7.1 ± 10	15.8 ± 13.6
White blood cell count (10^3/μl)	8.1 ± 1.9	11.1 ± 5.5	17.0 ± 10.6
Hemoglobin level (g/liter)	11.0 ± 1.6	7.4 ± 2.3	6.3 ± 2.2
Plasma creatinine level (μmol/liter)	36.3 ± 10.4	37.6 ± 8.9	61.7 ± 35
Geometric mean parasitemia (trophozoites/μl)	328[b]	55,872	35,789

[a] Data are presented as means ± standard deviations, except for parasitemia.
[b] Refers to those with asymptomatic parasitemia (n = 5).

TABLE 2. Characteristics of adult subjects in Indonesian study groups upon enrollment[a]

Variable[b]	HC (n = 43)	UM (n = 43)	SM (n = 70)
Age (yr)	27.4 ± 10.1	29.3 ± 9.3	26.7 ± 9.4
Wt (kg)	56.1 ± 7.2	56.6 ± 8.7	54.4 ± 8.1
% Male	50	64	82
White cell count (10^9/liter)	7.6 ± 2.8	7.2 ± 7.7	9.0 ± 4.1
Hb (g/dl)	12.9 ± 3.0	12.8 ± 1.9	9.6 ± 3.0
Plasma creatinine level (μmol/liter)	77.2 ± 13.0	89.8 ± 16.1	270.4 ± 266.2
Time from last meal (h)	12.0 ± 0.3	11.3 ± 5.7	16.5 ± 14.8
Chloroquine use prior to study enrollment [no. of patients (%)]		23 (53)	28 (40)
Duration of quinine treatment prior to enrollment (h)	0	3.1 ± 5.3	9.1 ± 11.6
ALT level (U/liter)	15.9 ± 6.3	29.4 ± 27.2	46 ± 37.7
HCO_3 level (mmol/liter)	28.8 ± 1.7	25.2 ± 2.2	25.4 ± 5.1
Geometric mean parasitemia (trophozoites/μl)	149[c]	4,136	10,605

[a] All values represent means ± standard deviations, except for parasitemia.
[b] Hb, hemoglobin; ALT, alanine aminotransferase.
[c] Applies only to those with asymptomatic parasitemia.

Reanalysis of arginine levels including the recently located samples does not change any interpretation made in the previous publication (14). However, for the total number of samples now available, the mean arginine level for HC children was significantly less (122 versus 103 μM), as was the mean arginine level for subjects UM (70 versus 56 μM). The mean arginine levels for children with CM (45.4 versus 45 μM) and the statistical association between plasma arginine concentration and case fatality remain unchanged. Amino acids were also measured in plasma collected from Indonesian adults, aged 14 to 60 years, in Papua, Indonesia. Study subjects had been enrolled in the following groups: (i) CM (children) and SM (adults), defined using modified WHO criteria (20); (ii) UM (children and adults) (1); and (iii) HC (adults and children) (1, 2). Plasma samples were collected upon enrollment in the study and were stored at −80°C. Some adults had received quinine prior to enrollment, and the duration of quinine therapy prior to sample collection was recorded. A second plasma sample was collected from adult SM survivors 3 days after enrollment but was unavailable for children and adults who died with SM (all deaths occurred within the first 3 days). All patients diagnosed with CM or SM were managed according to national protocols and treated with intravenous quinine. Institutional review board approval was given for the study, and informed consent was obtained from participants or their guardians.

Analytical methods. Samples were prepared as described previously (14) and derivatized with AccQFluor Reagent (Waters Corp.) according to the manufacturer's recommendations. A standard curve was prepared in a similar fashion. Alpha-aminobutyric acid was used as an internal control. Ten microliters of derivatized sample was used to quantify amino acid levels using reverse-phase high-performance liquid chromatography (HPLC) with gradient conditions and fluorescence detection. Three solutions were used to generate the gradient at a flow rate of 1 ml/minute: solution A (AccQFluor Eluent A [Waters Corp.]), solution B (100% HPLC-grade acetonitrile), and solution C (double-deionized water). The gradient was generated according to the manufacturer's recommendations (Waters Corp.). Intra-assay variability was 0.65%, and interassay variability was 1.4%. With this method, we were able to quantify levels of basic amino acids (arginine, ornithine, and lysine), aromatic amino acids (phenylalanine and tyrosine), and neutral amino acids (histidine, leucine, and isoleucine). There was no difference in the phenylalanine levels of HC adults and those of HC children; therefore, the normal range for plasma phenylalanine was generated by combining all HC data from Tanzanian and Indonesian groups and is based on the mean ± 2 standard deviations. For phenylalanine, this range was 40 to 84 μM. The normal ranges generated by our method conformed closely to those established by the Biochemical Genetics Section, ARUP Laboratories, University of Utah, where analysis was performed using ion-exchange chromatography with detection using spectrophotometry after a reaction with ninhydrin. The small volumes of plasma available from the majority of our subjects precluded amino acid analysis by this method. Plasma samples from healthy Caucasians and study subjects with sufficient volume were analyzed both by our method and at the Biochemical Genetics Section, ARUP Laboratories, University of Utah. Reliability, denoted R, between the two amino acid assays was calculated using the intraclass correlation coefficient. In the calculation, the assay method was considered a fixed effect, since inference applies only to these two specific methods (8). A generally accepted rule of thumb for interpreting the reliability coefficient is as follows: $R < 0.4$ represents poor reliability, $0.4 \leq R \leq 0.75$ represents fair to good reliability, and $R > 0.75$ represents excellent reliability. The reliability coefficients between the two amino acid assays were as follows: phenylalanine, R = 0.99; tyrosine, R = 0.84; arginine, R = 0.77; ornithine, R = 0.81; lysine, R = 0.71; histidine, R = 0.55; leucine, R = 0.70; and isoleucine, R = 0.66, all of which are acceptable levels of reliability. Based on these results, we were confident in using HPLC for amino acid analysis.

Statistical methods. Fisher's exact test was used to compare proportions. Continuous variables were compared using an independent-groups t test if normality assumptions were met; otherwise, groups were compared using the Mann-Whitney U test. Longitudinal data were analyzed using a paired t test. Multiple linear regression was used to control for confounding variables. Variables with extreme skewness or kurtosis were log transformed to meet the normality assumption for use in these linear regression models. All statistical analyses were performed using Stata 8.0 software. P values of <0.05 were considered to be statistically significant.

RESULTS

Of the 191 children in the original Tanzanian study (1), 126 (38/50 [76%] HC, 36/55 [65%] with UM, and 52/86 [60%] with CM) had a sufficient amount of plasma available for amino acid analysis. Of the 52 children with CM in this study, 20 died and 32 survived (38% case fatality). The case fatality in the original study was 30% (26/86) (1). Patient characteristics were published previously (1, 14) and are listed in Table 1 for our subset. Fourteen children with CM had concomitant severe malarial anemia, but none had acute renal failure. In a separate study of Indonesian adults with SM, plasma samples from 156 adults (43 HC, 43 UM, and 70 SM samples) were available for sampling. Adult patient characteristics at enrollment are listed in Table 2. In the SM group, 57 survived and 13 died. Fifty-eight percent of adults presenting with SM had CM, 34.3% had renal failure, and 2.7% had severe anemia. Seven (9.6%) patients with SM had both renal failure and CM, five (71%) of whom died. No deaths occurred in either adult or childhood UM groups.

TABLE 3. Plasma amino acid levels in Tanzanian children[a]

Amino acid	Mean amino acid level (μM) ± SD			
	Normal range[b]	HC (n = 38)	UM (n = 36)	CM (n = 52)
Phenylalanine	30–80	61 ± 13	118 ± 52[c]	177 ± 81[c,d]
Tyrosine	30–120	50 ± 10	53 ± 13	85 ± 47[c,d]
Phe/Tyr ratio		1.3 ± 0.32	2.4 ± 1.2[c]	2.2 ± 0.9[c]
Arginine	40–160	100 ± 32	56 ± 22[c]	45 ± 15[c]
Ornithine	20–135	64 ± 24	37 ± 15[c]	34 ± 12[c]
Lysine	80–250	151 ± 31	94 ± 28[c]	108 ± 47[c]
Histidine	50–130	80 ± 25	61 ± 18[c]	65 ± 20[c]
Leucine	60–230	116 ± 36	118 ± 51	139 ± 41[c,d]
Isoleucine	30–130	63 ± 20	71 ± 38	54 ± 18

[a] Groups compared using Wilcoxon-Mann-Whitney test with significance defined as a P value of <0.013, given multiple groups.
[b] Normal range as established by the Biochemical Genetics Section, ARUP Laboratories, University of Utah.
[c] Significantly different from HC ($P < 0.013$).
[d] CM significantly different from UM ($P < 0.013$).

In addition to decreased arginine levels relative to controls reported previously (14), in children, there were statistically significant decreases in ornithine, lysine, and histidine levels in all clinical malaria groups compared to levels in HC (Table 3). Despite significant differences, the mean plasma levels of these amino acids were within the normal ranges established for screening for inherited metabolic diseases in children. By contrast, phenylalanine was the only elevated amino acid in the malaria groups, with the mean greater than the upper limit of the normal range (Fig. 1 and Table 3). The proportion of children with abnormal phenylalanine levels was significantly greater in the CM group (49/52 [94%]) than in the UM group (27/36 [75%]) ($P = 0.013$). Likewise, the proportions in adults with hyperphenylalaninemia was higher in the SM group (53/70 [76%]) than in the UM group (19/43 [44%]) ($P = 0.001$). In contrast, only 2 of 38 HC children and 0/43 HC adults had elevated phenylalanine levels. The degree of hyperphenylalaninemia correlated with disease severity, with levels in CM and SM groups significantly greater than those in the respective HC and UM groups (Fig. 1a and b). This association remained after controlling for age, weight, duration of fasting, use of chloroquine prior to admission, duration of quinine use prior to sample collection, biochemical hepatitis, and renal function using linear regression. There was no association between phenylalanine levels and CM case fatality in children;

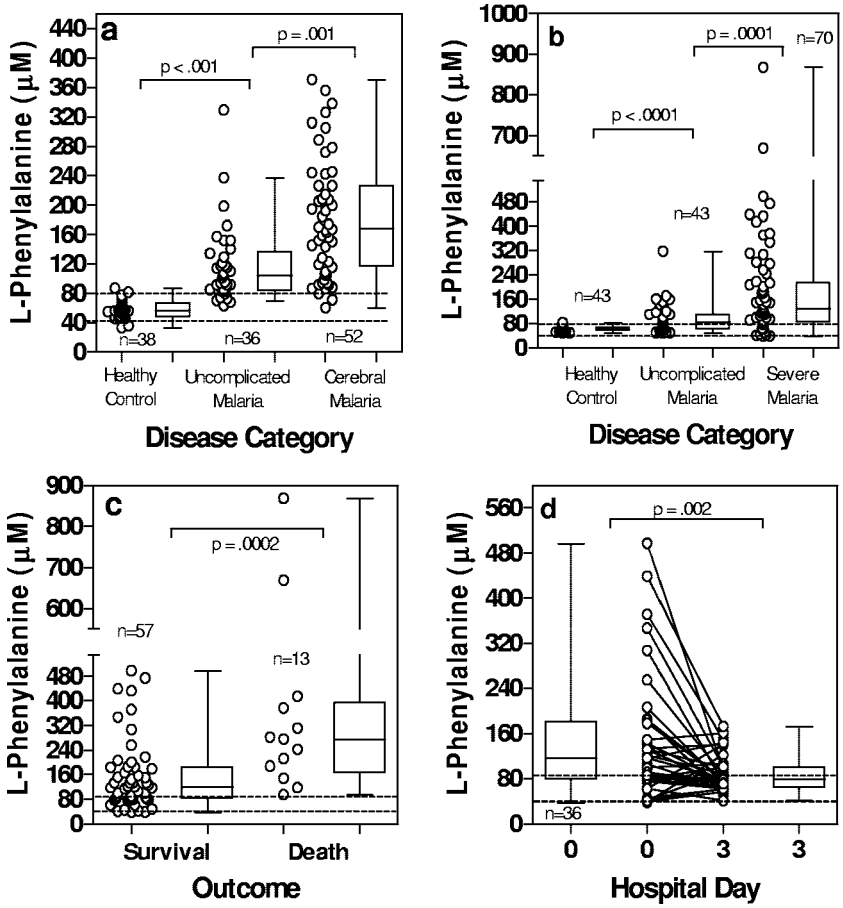

FIG. 1. Plasma phenylalanine concentrations in severe malaria. (a) Day 0 levels in Tanzanian children with CM. (b) Day 0 levels in Indonesian adults with SM. (c) Day 0 levels in Indonesian adults by outcome, death (median [95% confidence interval], 275 [163 to 399] μM) versus survival (median [95% confidence interval], 118 [90 to 140] μM). (d) Longitudinal data for Indonesian adults on day 0 (median [95% confidence interval], 116 [86 to 133] μM) and day 3 (median [95% confidence interval], 76 [70 to 90] μM) of the study. Box plot, median and interquartile range; whiskers, maximum/minimum range; dotted lines, phenylalanine means ± 2 standard deviations (normal range) based on 80 HC samples. Differences between means were compared using a Wilcoxon-Mann-Whitney test. Longitudinal data were compared using a paired two-tailed t test.

however, levels were higher in SM case fatalities than in survivors (Fig. 1c). Phenylalanine levels did not differ between adults with CM and those with other forms of SM or between adults with and without renal failure. Phenylalanine levels were significantly decreased by day 3 of treatment in SM survivors but did not completely normalize (Fig. 1d). Hyperphenylalaninemia was not correlated with coma score in children with CM or in adults with SM.

Tyrosine, the single, immediate product of phenylalanine metabolism by hepatic phenylalanine hydroxylase, was increased in CM (Table 3) and SM (mean ± SD, 81 ± 46 μM) groups only. The significance of this finding is unclear, as tyrosine levels in CM and SM groups were within the normal range. Moreover, provoked hyperphenylalaninemia in healthy adults leads to a mild elevation of plasma tyrosine levels (19). To better define the mechanism of hyperphenylalaninemia, we calculated the phenylalanine/tyrosine ratio, a sensitive measure of phenylalanine hydroxylase activity. This ratio has been used for the detection of heterozygous carriers of phenylketonuria mutant genes (9), as impairment of phenylalanine hydroxylase leads to ratios above normal (0.8 to 1.2) (3). The phenylalanine/tyrosine ratio was increased in all groups with clinical malaria but was not different between mild and severe disease (Table 3). This was due in part to increases in plasma tyrosine levels in the CM and SM groups.

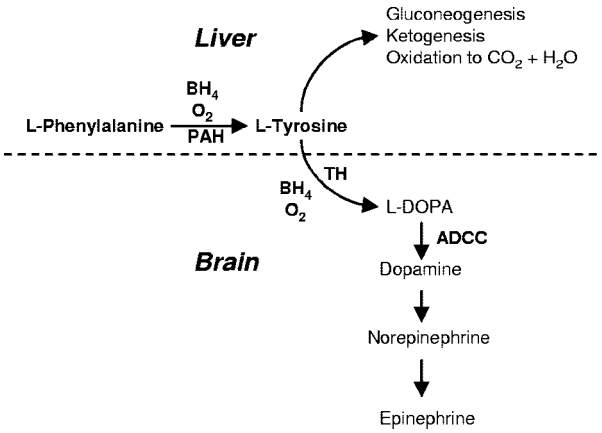

FIG. 2. Relationship of tetrahydrobiopterin-dependent aromatic amino acid metabolism to biogenic amine neurotransmitter synthesis. The plasma concentration of L-phenylalanine is regulated by changes in the activity of hepatocyte phenylalanine hydroxylase (PAH). Phenylalanine hydroxylase activity depends in part on hepatocyte synthesis of its obligatory cofactor, tetrahydrobiopterin (BH_4). Tyrosine, the phenylalanine hydroxylase reaction product, enters the blood and is taken up by neurons for further synthesis to L-DOPA (3,4-dihydroxyphenylalanine) by tyrosine hydroxylase (TH), another BH_4-dependent enzyme. L-DOPA serves as the precursor for synthesis of several biogenic amine neurotransmitters (dopamine, norepinephrine, and epinephrine), beginning with the enzyme L-amino acid decarboxylase (ADCC).

DISCUSSION

The causes and sequelae of chronic hyperphenylalaninemia have been well characterized in children with inborn errors of phenylalanine metabolism. However, less is known about the physiologic consequences of acute perturbations in phenylalanine metabolism, especially in infectious diseases. Hyperphenylalaninemia and an elevated phenylalanine/tyrosine ratio in children with falciparum malaria have been observed in one previous study in which four children with CM had elevated phenylalanine concentrations (7) similar to the levels we measured. Inhibition of phenylalanine metabolism has also been reported in sepsis of various etiologies (4, 6, 21) but to a lesser degree than that observed in CM. Despite these observations, the mechanism by which hyperphenylalaninemia develops in acute infections has not been established. Release of phenylalanine into the circulation from skeletal muscle catabolism may be an important factor. Under normal circumstances, excess phenylalanine (e.g., phenylalanine loading test) leads to substrate-level regulation of hepatic phenylalanine hydroxylase activity with consequent lowering of plasma phenylalanine levels within a narrow range (40 to 84 μM). This enzyme converts phenylalanine to tyrosine in an oxygen- and pterin-dependent reaction (Fig. 2). The elevated phenylalanine/tyrosine ratio in malaria suggests that hyperphenylalaninemia results from impaired phenylalanine hydroxylase activity in the liver.

Hyperphenylalaninemia in malaria may have important physiologic consequences. Two examples come from inborn errors affecting aromatic amino acid metabolism (e.g., phenylketonuria and dopamine-responsive dystonia [DRD]) that lead to neurological abnormalities. These genetic diseases impair phenylalanine hydroxylase activity by different mechanisms, and both cause chronic neurological disease in children. Phenylketonuria arises from inherited mutations in the phenylalanine hydroxylase gene that lead to persistent elevations in plasma phenylalanine levels up to 50 times greater than normal. High concentrations of plasma phenylalanine lead to phenylalanine accumulation in the brain, which is neurotoxic (12). In contrast, DRD is caused by a tetrahydrobiopterin deficiency from loss-of-function mutations in the GTP cyclohydrolase gene, the rate-limiting enzyme for tetrahydrobiopterin biosynthesis. Tetrahydrobiopterin is a required cofactor not only for phenylalanine hydroxylase but also for other enzymes (i.e., neuronal NO synthase, tryptophan hydroxylase, tyrosine hydroxylase, and dopamine β-hydroxylase) essential for brain neurotransmitter biosynthesis (16) (Fig. 2). DRD manifests with mild hyperphenylalaninemia, decreased monoamine neurotransmitter metabolite levels in the spinal fluid, and neurological dysfunction independent of plasma phenylalanine levels (18).

An underlying genetic phenylalanine hydroxylase abnormality as a cause of hyperphenylalaninemia in malaria is unlikely, since we would not have expected a rapid correction of plasma phenylalanine levels and the phenylalanine/tyrosine ratio (data not shown) upon clinical improvement with conventional treatment. Moreover, the degree of hyperphenylalaninemia that we observed in malaria is not consistent with the extreme elevations seen in phenylketonuria. Given the phenotypic similarities between children with DRD and children with CM (e.g., somnolence, recurrent seizures, motor dystonia, abnormal posturing, and autonomic instability) (18), we favor an acquired tetrahydrobiopterin deficiency as a more plausible explanation for hyperphenylalaninemia in malaria.

There is minimal passage of tetrahydrobiopterin across the blood-brain barrier (13). Thus, neurotransmitter production requires local de novo synthesis of this cofactor. Brain tetra-

hydrobiopterin deficiency in CM, but not UM, is a possible explanation for the absence of neurological symptoms in individuals with UM and hyperphenylalaninemia. Tetrahydrobiopterin levels have been measured in the spinal fluid of African children with CM in two separate studies, revealing conflicting results. One study of Zambian children with CM revealed that low total biopterin (biopterin, dihydrobiopterin, and tetrahydrobiopterin) in cerebral spinal fluid correlated with deep coma (22). Another study in coastal Kenya reported elevated tetrahydrobiopterin levels in subjects with CM that were more than 10 times higher than those measured in Zambian children (5). The discrepant results highlight the difficulties in measuring biopterin metabolites in biologic fluids (10), especially in clinical studies of malaria in developing countries (11). Hence, whether systemic and/or central nervous system tetrahydrobiopterin deficiency is present in malaria remains an open question.

In conclusion, we have shown that children with CM and adults with all forms of SM have disordered phenylalanine metabolism, which may result from impaired hepatic phenylalanine hydroxylase activity. Whether hyperphenylalaninemia is associated with brain neurotransmitter and pterin abnormalities in CM has therapeutic implications (such as adjunctive tetrahydrobiopterin replacement therapy) and warrants further investigation.

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